Heterotransplantation Model of Human Malignant Melanoma

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SUMMARY

A heterotransplantation model of human melanoma has been established in immunosuppressed Wistar-Furth rats. Heterotransplantation is accomplished by inoculating live cultured melanoma cells subcutaneously in Wistar-Furth neonates immunosuppressed with antithymocyte serum. Two different tumor cell lines (BeRo and HaLe) have been transplanted, and the BeRo line has been maintained through serial transplantation for up to 12 generations. The transplanted BeRo tumor line produced pigment, and both lines exhibited an aneuploid karyotype with the banding characteristics of human chromosomes. Multiple pulmonary metastases have been noted in one animal bearing a s.c. (BeRo) tumor transplant.

Membrane immunofluorescence studies with autologous and allogeneic human sera revealed discrete sites of cross-reacting antigen(s) on cell membrane of the melanoma cells both prior to and after heterotransplantation. Appreciable augmentation in the antigenicity of cell membrane characterized by an increased number of sites of immune complexes or by a complete ring of fluorescence was observed with the animal-passaged cells, as opposed to fewer sites of immune complexes on the cultured cells. Sera from several tumor-bearing rats also revealed circulating antibody directed against antigen(s) on transplanted melanoma cells, and often demonstrated cross-reaction with cells derived from several other melanoma cell lines. Further studies are underway to determine the nature and specificity of the circulating antibody.

The heterotransplantation model provides a close approximation of an in vivo situation that can be conveniently used in cytokinetic, chemotherapeutic, or other relevant biological studies concerning human melanoma.

INTRODUCTION

No satisfactory heterotransplantation system of human melanoma is currently available. Direct implantation and serial transplantation of human acute lymphoblastic leukemia (1), Burkitt's tumor (2) in hamsters, and temporary heterotransplantation of a breast carcinoma cell line (3) have been reported. Considering the wide scope of a heterotransplantation system in immunological and other relevant experimental studies, the study described below was undertaken to establish a heterotransplantation model of human melanoma. Live cultured human melanoma cells were chosen for s.c. transplantation in immunosuppressed WF rats. After the successful “take” of a heterotransplant, attempts were made to establish a serial transplantation technique, and the effect of such heterotransplantation on cell surface antigen(s) was studied.

MATERIALS AND METHODS

Tissue Culture and Heterotransplantation Technique. Sterile specimens of tumors obtained from biopsies are finely minced with curved scissors. The resultant mince and the cell suspension are washed in Ham's F-10 medium (Grand Island Biological Co., Grand Island, N. Y.) and resuspended in the same medium containing 100 units of penicillin per ml and 100 mg of streptomycin per ml, supplemented by 20% fetal calf serum. Tissue cultures are grown in monolayer in T 60 plastic flasks (Falcon Plastics, Oxnard, Calif.) or in 1-liter glass Blake bottles (Belco, Glass, Inc., Vineland, N. J.) at 37°.

Cultures are fed 3 times weekly and trypsinized with 0.25% trypsin when such cultures grow confluent.

For a steady supply of recipient animals, a breeding colony of WF rats is maintained in our laboratory (adult WF rats are purchased from Microbiological Associates, Inc., Bethesda, Md.). Heterotransplantation is carried out by s.c. inoculation of washed cell suspension (1 X 10^7 cells in 0.10 ml of 0.9% NaCl solution containing 100 units of penicillin per ml and 100 mg of streptomycin per ml) through a 21-gauge needle near the ventral or dorsal axillary space of WF neonates at 1 day of age. Prior to inoculation, all recipient neonates are given 0.05 ml of 1:2 dilution of either antithymocyte serum prepared in our laboratory or antilymphocyte serum purchased from a commercial source (Microbiological Associates, Inc.). After tumor cell inoculation, the recipient neonates are given further ATS treatments 3 times a week until tumor growth is palpable at the site of inoculation, after which, an attempt is made to taper off ATS administration by increasing the intervals between these treatments.

For serial passage of the transplant, the tumor-bearing animal is sacrificed and the tumor explant free of fatty and necrotic tissue is minced. The resultant fine mince and single cells are washed and resuspended in 0.9% NaCl solution containing penicillin and streptomycin, and 0.1 ml of the...
suspension (containing approximately $1 \times 10^7$ cells) is inoculated in neonates similarly treated with ATS. Strict aseptic measures are always observed during the transplantation procedure.

**Membrane Immunofluorescence Test.** Indirect membrane immunofluorescence technique has been used to detect membrane-bound antigen in melanoma cells and circulating humoral immunity against such antigen. Trypsinized cells ($1 \times 10^5$) are incubated in microfuge tubes with test serum (in desired concentration) for 0.5 hr at 37°, after which the cells are washed in phosphate-buffered saline 3 times and incubated with a commercially available (Hyland Div., Costa Mesa, Calif.), fluorescein isothiocyanate-labeled anti-human (or anti-rat) globulin (1:20) for another 0.5 hr at 37°. The cells are again washed, resuspended in 20% glycerol, and mounted on glass slides and read for specific membrane immunofluorescence. A positive reaction is characterized by discrete sites of bright apple-green fluorescence or a complete ring of fluorescence along the cell membrane (Fig. 2). A diffuse bright green staining is considered indicative of cell death.

**RESULTS**

Two different melanoma cell lines (BeRo and HaLe) have been successfully transplanted in immunosuppressed WF neonates up to 12 generations. Subsequent in vitro subcultures from the tumor explants were easily accomplished, and tumor cells from such subcultures were retransplanted without difficulty. Gross bacterial contamination accounting for neonatal death was not observed in this study.

After successful initial transplantation, the BeRo tumor line has been serially transplanted in immunosuppressed WF neonates up to 12 generations. Subsequent in vitro subcultures from the tumor explants were easily accomplished, and tumor cells from such subcultures were retransplanted without difficulty. Gross bacterial contamination accounting for neonatal death was not observed in this study.

**Metastasis.** No metastases to the regional lymph nodes have been observed. However, bilateral pulmonary metastasis was noted in only 1 animal (of 66 tumor-bearing animals) at age 23 days carrying a s.c. tumor transplant (cell line BeRo) at the 2nd generation of serial passage. Multiple firm nodules, histopathologically confirmed to be metastatic melanoma, were felt macroscopically scattered throughout the lungs (Fig. 1c). Some areas of the lung were completely replaced by the tumor. The metastatic nodules revealed marked mitotic activity.

**Histopathology.** The tumor explant morphologically consisted of nodules or sheets of malignant cells surrounded at times by thin layers of fibroconnective septae containing small blood vessels (Fig. 1b). The nodules were composed of poorly differentiated cells with prominent nucleoli and exhibited marked mitotic activity. Pigment production was demonstrable by both hematoxylin and eosin staining and by Fontana Masson staining in the transplanted tumor line WF HaLe, while the WF BeRo line was amelanotic (Table 1). Degenerative changes and necrosis were seen at time of death.

**Chromosome Analysis.** Both the melanoma cells derived from long-term tissue culture and the animal-passaged cells exhibited aneuploid karyotype with banding characteristics of human chromosomes.

**Membrane Immunofluorescence.** Autologous and allogeneic sera from other melanoma patients gave positive reactions, characterized by discrete site(s) of fluorescent immune complexes (+ to ++ on a + to ++++ scale) on the cell surface of cells derived from tissue culture (Fig. 2a). However, when a panel of positive sera were retested on cells derived from the 1st or 2nd in vitro subculture of the tumor transplant in parallel with cells derived from long-term tissue culture, (not animal-passaged), the animal-passaged cells demonstrated a more intense (+++++) reaction (Fig. 2b). Furthermore, an increased number of fluorescent sites on the cell membrane or a complete ring of fluorescence all along the cell membrane was seen on the animal-passaged cells, as opposed to fewer such sites demonstrated on the cells that were not passaged through animal transplantation. Experiments conducted with incubation temperatures at 37° and 4° did not change characteristics of either reaction. Interestingly, as the animal-passaged cells were maintained through serial in vitro subculture, the animal-passaged cells exhibited a tendency to lose the complete ring of fluorescence on subsequent testing and reverted back to the pattern of fewer discrete sites of reaction. This phenomenon was consistently observed on repeated experiments.

Autogenous sera from both patients (from whom the tumor cell lines BeRo and HaLe were derived) and sera from the tumor-bearing animals were positive for antibody against cell surface determinants on the transplanted cells and often

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Passage level at time of transplantation</th>
<th>Pigment production</th>
<th>Karyotype</th>
<th>Metastases</th>
</tr>
</thead>
<tbody>
<tr>
<td>BeRo</td>
<td>51–57</td>
<td>+</td>
<td>Aneuploid</td>
<td>Multiple pulmonary metastases in 1 animal</td>
</tr>
<tr>
<td>HaLe</td>
<td>11–12</td>
<td>+</td>
<td>Aneuploid</td>
<td>None observed</td>
</tr>
</tbody>
</table>

*The in vitro subcultures derived from the explants of the transplanted tumors are designated as WF BeRo or WF HaLe.*
cross-reacted against several other melanoma lines in vitro. Sera from normal littermate controls were negative against both cell lines.

No appreciable difference in the character or intensity of immunofluorescence was noted when cultured cells were tested after trypsinization or after mechanical liberation with a rubber policeman.

DISCUSSION

Transplantation studies in animal tumor system(s) have undoubtedly made major contributions in cancer research. Transplantation experiments in humans are obviously not realistic possibilities, yet a limited number of autotransplantation and homotransplantation studies (3, 7) have provided important information in tumor immunology. Heterotransplantation with certain types of human tumors have been reported (1, 2, 5, 6). Similarly, although human melanomas have been successfully transplanted in immunosuppressed hamsters by Cobb (4), a heterotransplantation system with serial transplantation is not available. If available, such a system will provide unique opportunities in research. A considerable amount of research has been done on immunological aspects of human melanoma and lately interest has been generated in various forms of immunotherapy in this disease. A heterotransplantation system in human melanoma thus offers potential use of such a system in immunological and immunotherapeutic studies in human melanoma.

This study suggests that a heterotransplantation model in human melanoma is indeed possible, as it is clear from the study that human melanoma can be serially maintained through successive generations. The s.c. transplant offers distinct advantages over the so-called privileged sites (cheek pouch of hamsters, brain, i.p. implants, ascites form in other recipient animals), since a s.c. tumor readily lends itself for easy inspection, palpation, and accurate measurements.

Although the presence of tumor-specific antigen(s) in human tumors has not conclusively been proven yet, the existence of such antigen(s) nevertheless has generally been accepted. The heterotransplantation model offers further opportunity for studies concerning tumor specificity of cell surface antigen by appropriate absorption studies (particularly since tumor-bearing rats make antibody directed against cell surface determinants). Such absorption studies are currently under way in our laboratory.

The detection of increased antigenicity of the cell membrane of the animal-passaged cells manifested by an increased number of antigenic determinants or a complete ring of fluorescence, appears to be a real entity. This phenomenon does not appear to be similar to the phenomenon of temperature-dependent redistribution of cell surface antigen (8). The difference in reactivity is not only appreciable when experiments are conducted in parallel under identical conditions, but also from the fact that incubation conducted at various temperatures (4–37°C) does not alter either reaction. Nor is it due to possible alteration of membrane antigen, caused by trypsinization, as both the trypsinized and the mechanically liberated cells behave identically regarding their cell surface antigenic determinants. The consistency of the observations suggests that heterotransplantation imparts either a better expression (easier detection) or antigenic augmentation (actual increase) of cell surface antigen(s).

The heterotransplantation system thus may be used as a tool to restore, preserve, or augment cell surface antigen. Furthermore, this method of augmentation of cell surface antigen may have potential application in immunotherapeutic schema in human melanoma, if such tumor cells are proved to be immunogenic. The animal-passaged cells with augmentation of cell surface antigen then will be the ideal choice for active specific immunotherapy in this disease. Immunogenicity of such cells and cell products are currently under study in our laboratory. Beside immunological studies, the model offers a close approximation of an in vivo situation which can conveniently be utilized in cytokinetic, chemotherapeutic, and other relevant studies concerning human melanoma.

ACKNOWLEDGMENTS

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REFERENCES

Fig. 1. a, photograph of the transplanted HaLe tumor line, the arrow indicates growth of tumor along the needle track. Although the smaller nodule in the axilla appears as nodal metastasis, it was in fact histopathologically found to be tumor growth from the inoculum. In b the histopathology of the tumor explant is demonstrated (H & E, X 520), with pigment production shown by arrow. In c, multiple pulmonary metastasis (H & E, X 350) is shown in an animal bearing the transplanted BeRo line.

Fig. 2. Membrane immunofluorescent photomicrographs of melanoma cells grown in serial tissue culture (a), and after heterotransplantation (b). The cell in serial tissue culture (BeRo) demonstrates discrete areas of fluorescence (a), whereas a complete ring of fluorescence is observed on the cell derived from transplanted tumor explant WF BeRo (b). In a, tumor cell with a greater than usual number of immune complexes was chosen for better illustrative purposes. The number of such complexes on the cells derived from tissue culture (not animal passaged) is usually less than that is shown in a.